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14. ABSTRACT: Our objective is to develop a realistic preclinical model of prostate cancer by developing methodology that supports the survival, growth and differentiation of primary cultures of prostate cells in mice. During year 1, we focused on the method of implantation and the implantation site as the most critical elements in achieving this goal. In light of increasing evidence that stem cells are the only cells in cancers that have potential for sustained self-renewal, we now believe that primary cultures in fact will never be capable of forming tumors, regardless of the method of implantation, unless they contain stem cells. Analysis of the primary cultures established by our routine protocols suggested that stem cells were not present. Therefore, we devoted year two of this project to developing new methodology to establish prostate cancer stem cells in primary culture. Key steps included defining a protocol to isolate single, viable cells that retained cell surface antigens from fresh human cancer specimens, isolating distinct subpopulations of single cells expressing putative stem cell antigens, and determining the culture conditions required for single cells to attach and proliferate. These achievements now allow us to return to our original objective of defining optimal in vivo conditions, but with primary cultures that have the requisite stem cells necessary for tumor formation.

15. SUBJECT TERMS
In vivo model development, primary cultures, epithelial and stromal Cells, preclinical model, stem cells

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INTRODUCTION

Currently available models of prostate cancer do not realistically predict activity of experimental therapeutic agents in clinical trials. The objective of our proposed research is to develop a model system that will allow the translation of *in vitro* results to an *in vivo* environment and provide a more realistic preclinical model of prostate cancer than currently exists. Primary cultures, which provide a key *in vitro* model of normal and malignant prostate biology, could fulfill this objective if we can devise a means by which they can be maintained *in vivo* and express appropriate structural and functional differentiation. Our past studies showed that primary cultures transplanted into *nude* mice via standard subcutaneous injection methods rapidly became squamous. We hypothesized that hypoxia is the factor that triggers inappropriate squamous formation that prevents appropriate growth and prostate-specific differentiation of primary cultures *in vivo*. Our experimental plan was to circumvent hypoxia by transplanting cells on a unique gas permeable membrane under the highly vascularized subrenal capsule of the mouse. Our aims were (1) To transplant primary epithelial cell cultures grown on OptiCell™ membranes under the renal capsule of *nude* mice, (2) to transplant primary stromal cell cultures grown on OptiCell™ membranes under the renal capsule of *nude* mice, and (3) to transplant co-cultures of epithelial and stromal cells on OptiCell™ membranes under the renal capsule of nude mice.

In Year One, as described in the first annual progress report, we carried out those Aims and encountered some problems related to the nature of the OptiCell™ membranes. We concluded that we would have to identify a matrix other than OptiCell™ membranes on which to transplant primary cultures into mice. However, as we developed our experimental strategy for Year Two, we concluded that we could not ignore the developing field of cancer stem cells. Cancer stem cell theory posits that a rare population of functionally distinct cancer cells possesses the extensive self-renewal potential necessary to create a tumor; these are cancer stem cells (CSCs) [1]. Progress in identification of CSCs in other solid tumors including those in the breast and brain has prompted strong belief that prostate cancer is a stem cell disease. Cancer stem cell theory further states that only therapies targeting CSCs will effectively render the tumors unable to maintain themselves or grow, thus effecting a cure.

Accordingly, we now believe that our original hypothesis, that hypoxia is the factor limiting growth of primary cultures *in vivo*, is incomplete. While hypoxia may indeed be a critical inhibitory element in the microenvironment, we now believe that primary cultures will never grow *in vivo* unless they contain stem cells. Therefore, in Year Two, we devoted our studies to determining whether our primary cultures as historically established do contain a subpopulation of stem cells and, if not, to identifying conditions that permit establishment and growth of stem cells in primary cultures. Our results are described below. Based on our progress this year, we expect in Year Three to return to our *in vivo* studies but with primary cultures that contain at least a subpopulation of stem cells.

BODY

Our first designated task was to transplant primary epithelial cell cultures grown on OptiCell membranes under the renal capsule of nude mice (months 1-12). Our specific

goals were to **(a)** prepare epithelial cell cultures on OptiCell membranes in vitro, **(b)** characterize epithelial cells grown on OptiCell membranes in vitro, **(c)** transplant epithelial cells grown on OptiCell membranes in vivo, and **(d)** characterize epithelial cells grown in vivo. We accomplished all components of this aim, as described in the first annual progress report (February, 2005). We concluded that the OptiCell membranes were not suitable as an implantation platform because they caused an undesirable inflammatory response. The membranes, composed of a proprietary plastic, are rather rigid and presumably cause physical trauma to the kidney, hence the inflammatory reaction and scar formation.

Our second aim was to transplant primary stromal cell cultures grown on OptiCell™ membranes under the renal capsule of nude mice (months 13-20). Our specific goals were to **(a)** prepare stromal cell cultures on OptiCell membranes in vitro, **(b)** characterize stromal cells grown on OptiCell membranes in vitro, **(c)** transplant stromal cells grown on OptiCell membranes in vivo, and **(d)** characterize stromal cells grown in vivo. Although our original intent had been to not initiate studies with stromal cells until Year Two, we decided to carry out experiments in conjunction with the epithelial cells in Year One, and these results were also described in the first annual progress report. Histologic analysis of the implanted membranes revealed the same phenomenon as noted in the experiments with epithelial cells. Even the OptiCell membrane itself with no cells caused inflammation and extensive scar formation, as was also seen with the membranes carrying cells. This validated our conclusion from the previous studies that the membranes acted as an irritant in the kidney and this property precludes their utility as a platform for implantation of cell cultures.

Our third aim was to transplant co-cultures of epithelial and stromal cells on OptiCell™ membranes under the renal capsule of nude mice (months 21-36). Our specific goals were to **(a)** co-culture epithelial and stromal cells on OptiCell membranes in vitro, **(b)** characterize co-cultures of epithelial and stromal cells grown on OptiCell membranes in vitro, **(c)** transplant co-cultures of epithelial and stromal cells on OptiCell membranes in vivo, and **(d)** characterize co-cultures of epithelial and stromal cells in vivo. Although we had not planned to initiate co-culture experiments until Year 3, we decided to start some of these experiments since we were already working with epithelial and stromal cells in Aims 1 and 2. Carrying out this Aim required in vitro studies to first identify optimal co-culture conditions, which were described in the first annual progress report. We did not attempt to implant any co-cultures on OptiCell membranes into nude mice given the problem with inflammation that we encountered in Aims 1 and 2.

At the end of Year One, we concluded that OptiCell membranes would not provide a suitable platform for implantation of primary cultures of prostatic cells under the renal capsule of nude mice. We expected to devote Year Two to identifying and testing other substrates for implantation. However, as alluded to in the Introduction, we have postponed those studies to Year Three. Instead, we devoted Year Two to testing methodology for the primary culture of prostate cancer stem cells. In Year Three, we will return to our goal of identifying an optimal method to grow primary cultures *in vivo*, except that our primary cultures will now contain the requisite stem cells. Our results are as follows:

(1) Search for presence of stem cells in primary cultures established according to standard methodology. If our pre-existing primary cultures contained stem cells, then we would be able to directly continue with *in vivo* studies. This does not appear to be the case, according to several criteria. First, one attribute of stem cells is infinite self-regeneration. Therefore, stem cells should be immortal. Our primary cultures, even those derived from cancers, have a finite lifespan and have never given rise to an immortal cell line. Thus, our primary cultures do not possess the characteristic infinite self-regeneration potential of stem cells. Secondly, stem cells often possess the ability to undergo anchorage-independent growth. Our primary cultures also do not exhibit this stem-related characteristic. Thirdly, stem cells typically express unique cell-surface antigens. CD133 has been implicated as a prostate epithelial stem cell antigen [2]. Immunostaining of our primary cultures, even very shortly after establishment, did not show any CD133-positive cells. On the basis of these findings, we concluded that primary cultures established by our standard methods are unlikely to contain stem cells. We then initiated a series of experiments aimed at altering our traditional primary culture methodology in order to establish primary cultures containing stem cells.

(2) Isolation of single cells from human prostate cancer tissues. Stem cells are often identified by the expression of specific cell surface antigens and sorted by flow cytometry. Therefore, in order to culture stem cells, it will be necessary to culture single cells. This is challenging for cells originating from glandular epithelium such as that of the prostate, since such cells prefer to be maintained as acini and attach as an aggregate. Our standard protocol for establishment of primary cultures involves digestion of tissues to acini, but not to single cells, because we have found that single cells do not attach or grow well.

Subsequently, we proceeded to optimize a protocol to generate a good single cell suspension from prostate cancer tissue. The key is to identify optimal enzymatic and mechanical techniques that break intercellular bonds but do not kill cells or digest cell surface proteins, which we will use to separate putative stem cells from the other cell populations. The optimal protocol that we developed involves a 2-4 hr digestion of minced tissue with medium containing high concentrations of collagenase I and hyaluronidase to release prostatic acini, and a short (5-10 min) digestion with 0.2% trypsin/0.2% EDTA to release single cells from the acini. We typically obtain an average of 1-2 x 10^5 prostatic cells/0.1g tissue. The trypsinization doesn't destroy cell surface antigens as shown by immunolabeling with antibody against epithelial cell-specific antigen (ESA) (Fig. 1).

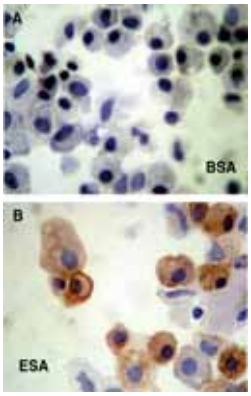


Fig.1. Immunolabeling of cells dissociated from fresh tissue by collagenase and trypsin digestion using antibody against ESA. Single cells were spun down on the surface of positively charged slides using cytospin 2 centrifuge. Cells were then fixed in 2% paraformaldehyde, and stained with a monoclonal antibody against human ESA. A biotinylated rabbit anti-mouse IgG was used as the secondary antibody. ABC reagent and DAB substrate were then used to detect the present of ESA. (A) Cells stained with 1% BSA as control showed no staining, demonstrating the specificity of the labeling procedure. (B) Cells stained with 2 µg/ml ESA antibody showed brown staining in a fraction of the cells, indicating an epithelial identity of these cells. Those that weren't stained presumably included blood cells, stromal cells, and neuroendocine cells. Note that the staining signal in epithelial cells was present both on the cell surface and in the cytoplasm. The cytoplasmic staining is likely due to slight permeabilization of the membrane by the fixation procedure.

This is also evident by flow cytometry analysis after staining with allophycocyanine (APC)-conjugated ESA antibody (Fig. 2). Note that the percentage of ESA^+ cells (20.3%) is low in the sample because there are large numbers of blood cells, especially the erythrocytes.

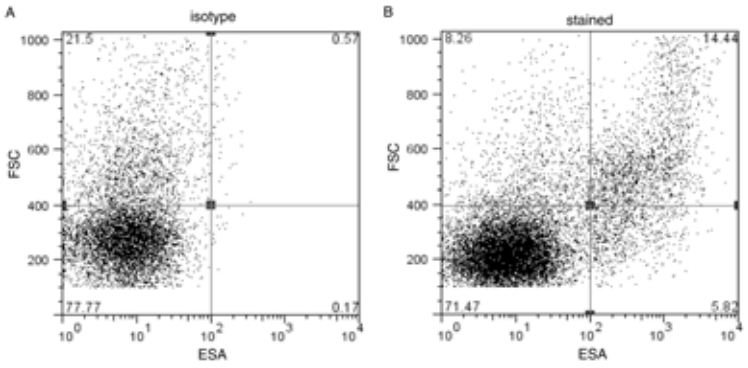


Fig.2. Analysis of ESA expression in cells dissociated from fresh tissue by collagenase and trypsin digestion using flow cytometry. Single cell suspension was incubated with mouse IgG first to block non-specific binding. Control cells (A) were incubated with isotype IgG conjugated with APC, and experimental cells (B) were stained with APC-conjugated monoclonal antibody against ESA at a concentration of 5 µg/ml. PI (1µg /ml) was used to gate out dying/dead cells.

CD133 is the most promising prostate stem cell marker currently under investigation. Collins's group showed that it was expressed by 0.7% of prostate cells from normal tissue using flow cytometry, and on a rare population of basal cells in tissue sections using immunohistochemistry [3]. We examined the expression of CD133 in single cells freshly dissociated from prostate cancer tissue using the protocol described above and flow cytometry. Single cells generated from a fresh cancer specimen after surgery were stained with Phycoerythrin (PE)-conjugated CD133 antibodies, AC133 and 293C3, each recognizing an epitope of the CD133 antigen. Cy7PE-conjugated anti-CD45 was used to distinguish leukocytes from other cells. We tried to eliminate erythrocytes using an ammonium chloride-based lysis buffer, but it had an obvious detrimental effect on the viability of the prostatic cells. (In subsequent studies, we tested a different buffer which seemed to work well without deleterious effects on the cancer cells). As shown in Fig. 3, a distinct rare population (2.2%) of $CD133^+$ cells existed in the tumor specimen. The percentage of the $CD133^+$ cells was probably underestimated because the total number of cells included the erythrocytes although the leukocytes were excluded by CD45 expression. Compared to a normal tissue specimen, there was a more than 5-fold enrichment of $CD133^+$ cells in the cancer specimen, consistent with the hypothesis that CSCs arise from the dysregulation of self-renewal of normal stem cells and therefore CSCs are in a greater number than their normal counterparts. These results demonstrated that CD133 is expressed by a rare population of cells in prostate cancer, characteristic of stem cell markers. In vitro culture of the cells generated from fresh tissue in the standard serum-free medium that our lab uses for primary culture of prostatic epithelial cells resulted in a loss of $CD133^+$ cells (data not shown). Similarly, $CD133^+$ cells were not detected by flow cytometry in several primary prostate epithelial cell cultures established previously in our lab (data not shown). At this point, we don't know whether CSCs do not grow in the conditions that we use for primary cultures, or whether CSCs might still be present in primary culture but they lose expression of CD133.

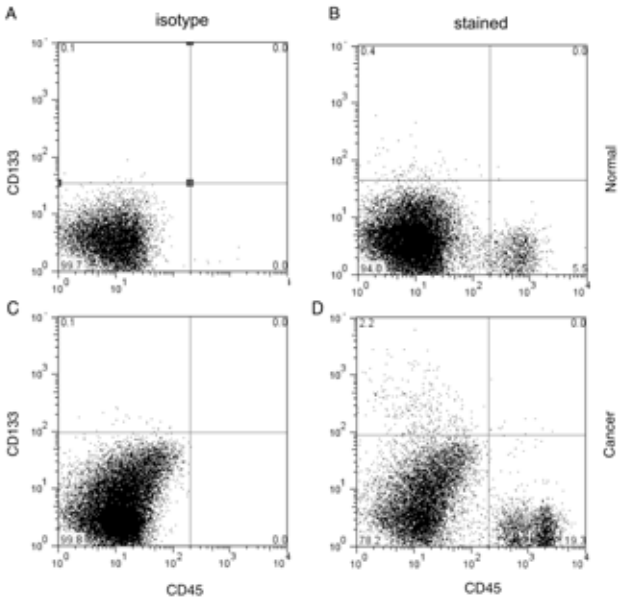


Fig.3. Flow cytometry analysis of CD133 expression in prostate cancer cells. Single cell suspension was generated from fresh normal or cancer tissue by collagenase and trypsin digestion. Cells were then incubated with mouse IgG to block non-specific binding. Control cells (A) were incubated with isotype IgGs conjugated with PE or Cy7PE, and experimental cells (B) were stained with PE conjugated monoclonal antibodies against CD133 at a concentration of 0.2 μg/ml together with a CY7PE conjugated CD45 (0.1 μg/ml). PI (1 μg/ml) was used to gate out dying/dead cells. Expression of CD133 was examined using LSR flow cytometer and data were collected using CellQuest and analyzed using Flowjo.

(3) Expression of GFP in prostate cells by lentivirus infection. Wnt signaling through the canonical β-catenin pathway has been shown to regulate stem cell renewal in several tissues [4]. For example, Wnt signaling promotes the maintenance of intestinal epithelial stem cells by promoting their proliferation and by coordinating the expression of adhesion molecules that regulate migration out of the SC niche in the intestinal crypts. Similarly, Wnt regulates the self-renewal of stem cells in the skin by regulating their proliferation and migration. Over-expression of β-catenin in hematopoietic stem cells also appears to promote stem cell self-renewal. Activation of TCF4-driven gene expression has been shown to be a direct downstream target event of the activation of Wnt signaling pathway, which is important for the maintenance of stem cells. For instance, in TCF4 knockout mice, the stem cell compartment of the small intestine is no longer present. Therefore, it is conceivable that stem cells in the prostate may also require TCF4-mediated gene expression to maintain their stemness. To select cells that have activated TCF4, we used GFP as a reporter and delivered TCF4-GFP, a construct in which GFP is linked to a promoter with three TCF4 binding sites, into cells by lentivirus infection. A control construct was used in which GFP is downstream of the same promoter except that the TCF4 binding sites are inactivated by mutations. Lentivirus carrying these constructs were produced in 293T cells, and concentrated by ultracentrifugation at a titer of 3.66×10^7 TU/ml. Fig. 4B shows that wild type TCF4 promoter facilitates high expression of GFP in HCT116, a colon cancer cell line that has constitutive β-catenin expression that activates TCF4. In contrast, the mutated promoter abolished GFP expression in HCT116 (Fig. 4C). In prostate epithelial cells digested from fresh tissues, lentivirus infection led to expression of GFP under the control of the phosphoglycerate kinase (PGK) promoter (Fig. 4D) at a level comparable to that in HCT116 cells (Fig. 4A), demonstrating the feasibility of delivering TCF4-GFP into these cells using lentivirus.

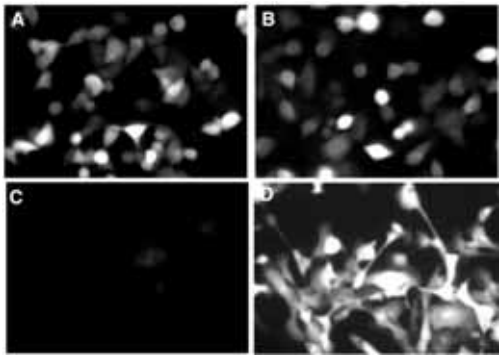


Fig.4. Fluorescence of GFP introduced by lentivirus infection. GFP was highly expressed in HCT116 (A), a colon cancer cell line constitutively expressing β-catenin, and prostatic cells generated from fresh tissue (D) under the control of a constitutively active PGK promoter. GFP expression driven by wild type TCF4 promoter (B) was also readily detectable in HCT116 cells. In contrast, mutations in TCF4 binding sites abolished GFP expression in HCT116 .

We infected single cells generated from a prostate cancer specimen with lentivirus carrying either wild type TCF4-GFP or mutated TCF4-GFP, and analyzed the GFP expression using flow cytometry. As shown in Fig. 5, 12.1% of the cells infected with wild type TCF4-GFP showed considerably higher level of GFP compared to cells infected with mutated TCF4-GFP, demonstrating the existence of a small population of cancer cells with activated TCF4-mediated gene expression. Whether CSCs are enriched in this population of cells needs further in vivo functional analysis to determine tumor initiating capability (which we will carry out in Year 3). We also examined CD133 expression in these infected cells, and found that the differences in CD133 expression between isotype control and antibody-stained cells were minimal, consistent with our previous observation that in vitro culture under standard conditions resulted in a loss of CD133 expression in CSCs or a loss of CSCs all together.

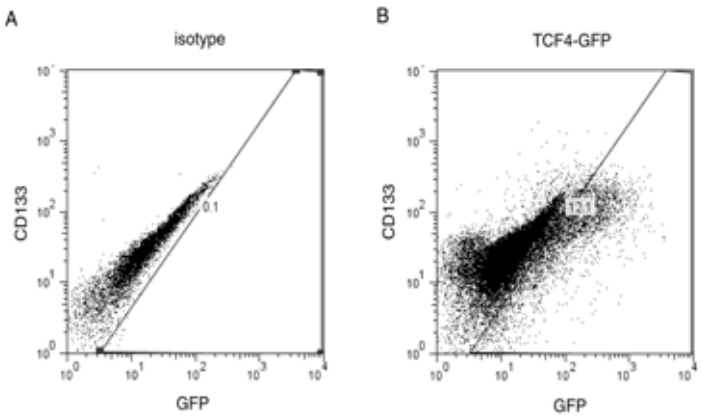


Fig. 5. Flow cytometry analysis of GFP expression under the control of TCF4 promoter in PCa cells. Single cells were plated and infected with lentivrus the next day. After three days, cells were collected by trypsinization, stained with PE-conjugated CD133 antibodies, and analyzed using LSR flow cytometer. (A) cells infected with mutated TCF4-GFP and stained with isotype IgG; (B) cells infected with wild type TCF4-GFP and stained with PE-conjugated antibodies against CD133.

(4) Primary culture of single cells. After demonstrating that we could create a population of viable single cells that retained cell surface antigens from digested fresh prostate cancers, our next goal was to identify conditions that would permit attachment and growth of these single cells *in vitro*. We first tested our standard primary culture conditions, which include collagen-coated dishes and the serum-free medium "Complete PFMR-4A" [5]. While these conditions are optimal for the attachment and growth of acini, single cells did not attach or grow in these conditions. We proceeded to test a number of other media and substrates and found success by using a feeder layer of stromal cells (mouse 3T3 cells) and a newly commercially available

defined medium from CellNTec (CnT-12). Complete PFMR-4A also supported attachment and growth of single cells in conjunction with a feeder layer; while growth in Complete PFMR-4A was perhaps not quite as good as in CnT-12, Complete PFMR-4A does offer the advantage that we know the constituents, unlike the commercial medium. Fig. 6 illustrates colonies that formed from single cells in CellNTEch medium with a feeder layer. While we do not yet know if any of these colonies were derived from stem cells, the ability to generate colonies from single cells represents the first required step towards culturing stem cells.

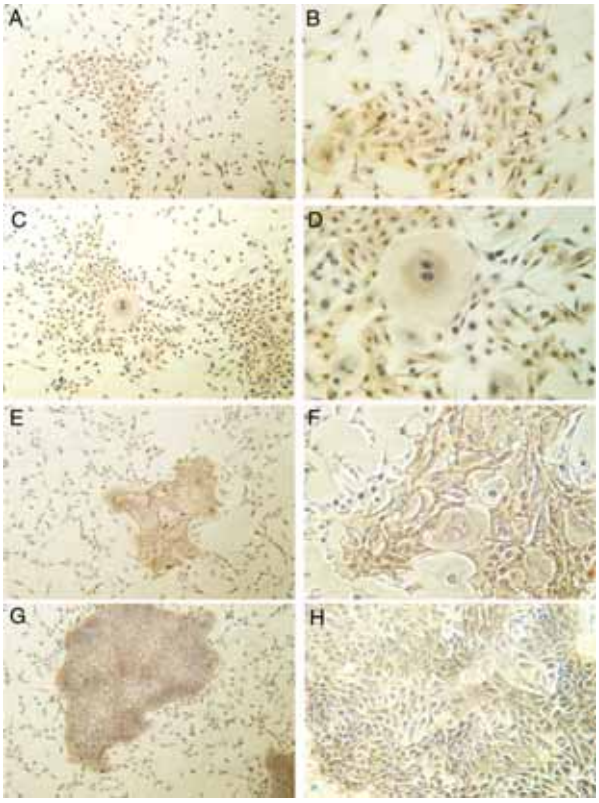
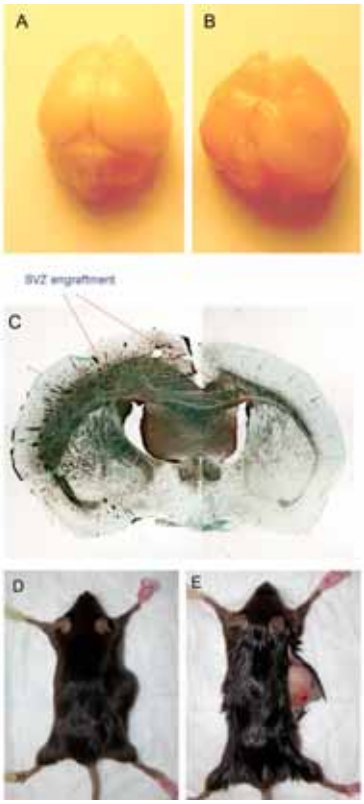


Fig.6. Colonies formed by single cells inoculated onto 3T3 feeder layers in CnT-12 medium (A-D) or Complete PFMR-4A medium (E-H). Colonies were stained with antibody against ESA.

(5) γc^-/RAG2$^-$ mice as an in vivo model for identification of CSCs. To obtain definitive evidence of the existence of CSCs, an *in vivo* functional analysis must be established. It is essential to determine whether a population of cells can initiate tumors *in vivo* in order to distinguish tumorigenic vs. non-tumorigenic cells. Various research groups have attempted to establish xenograft models of fresh, histologically intact human prostate cancer tissues in immunodeficient mice. The low engraftment rate experienced by these groups can be attributed to two main factors, the host environment and the grafting site. Several xenotransplantation models were developed based on severe combined immunodeficient (SCID) mice and their derivative, the non-obese diabetic (NOD)/SCID mouse model. The utility of the existing SCID mouse models is limited due to several disadvantages, including some "leakiness" that results in the appearance of mature B/T lymphocytes and immunoglobulins, residual natural killer (NK) cell activity, and a high rate of spontaneously developing thymomas that limit their lifespan. A new SCID mouse model has been developed by crossing mice lacking the common cytokine receptor γ chain for interleukin (IL)-2, IL-4, IL-7, IL-9, and IL-15 with mice lacking the recombinase activating gene-2 (RAG-2). The offspring has a stable phenotype characterized by the absence of all T and B cells and NK function. This novel immunodeficient mouse proved to be useful for studying xenotransplantation of human plasmacytoid dendritic cell precursors and human peripheral blood lymphocytes for the development of a severe acute graft-vs.-host disease model [6]. It also has been widely used by Dr. Weissman's lab and other stem cell investigators at Stanford with great success. The group has been astounded at the growth of enriched glioblastoma stem cells in this host. Glioblastomas, like prostate cancer, have traditionally proven very difficult to grow as xenografts in mice (Fig. 7A-C). Enriched stem-like cells from head and neck cancers (Fig. 7D, 7E), as well as from ovarian cancers, have also been growing well in the γc^-/RAG2$^-$ mice.

Therefore, we are hopeful that these mice will provide a particularly welcoming environment for prostate CSCs as well in our experiments in Year 3.



(Provided by Weissman lab)

Fig.7. Engraftment of primary human tumor cells in γc⁻/RAG2⁻ mice. Single cells generated from fresh cancer tissues were injected either into the brain or subcutaneously at the flank of the mice. (A) the brain from a mouse injected with control glioblastoma cells; (B) the brain from a mouse injected with glioblastoma cells enriched with stem cells; (C) engraftment of glioblastoma cells at the subventricular zone; (D), (E) mice injected subcutaneously with cancer cells from head and neck tumor.

We have made our first attempt to implant prostate cancer cells under the renal capsule of two γc⁻/RAG2⁻ mice. Cells were suspended in an equal volume of Matrigel and kept on ice until they were injected. The surgery was performed as described by Wang et al. [7] during a 20 min period for each mouse during which sterile practices were followed throughout. The mice were then kept in a designated area in the Stanford Research Animal Facility. Both mice survived the procedure, and there were no signs of infection.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that stem cells are unlikely to be present in primary cultures of prostatic epithelial cells established by standard methods
- Developed methodology to isolate viable single cells that retain cell surface antigens from tissues, a prerequisite for culturing stem cells
- Showed that a small population of cells expressing CD133, a putative prostate stem cell marker, is present in cells isolated from normal and malignant prostatic tissues
- Demonstrated successful infection with lentivirus of single cells isolated from tissues, a tool that can be utilized to isolate cells expressing Wnt signaling pathways associated with stem cells

- Using lentivirus, showed that a small population of cancer cells isolated from tissues can activate TCF4-mediated gene expression, a putative stem cell characteristic
- Identified novel culture conditions that permit clonal growth of single cells isolated from tissues, providing the basis for future culture of stem cells expressing CD133, TCF4-mediated gene expression, or other putative stem cell characteristics
- Identified γc^{-}/RAG2^{-} mice as a potentially superior *in vivo* host for the implantation of primary cultures containing stem cells in Year Three

REPORTABLE OUTCOMES

None.

CONCLUSIONS

Science is dynamic, and we have had to modify our original hypothesis as new information has developed. Our original premise was that primary cultures of prostate cancer cells would be capable of tumor formation *in vivo* if provided with the appropriate environment. We hypothesized that the appropriate environment would be under the renal capsule, where hypoxia would not be present and would not provoke growth-limiting, inappropriate squamous differentiation. We know believe that hypothesis to be too simplistic, because it did not take into account recently accumulating knowledge regarding the existence and characteristics of cancer stem cells. Accordingly, our hypothesis must now posit that growth *in vivo* will rely not only on the nature of the host environment but also on the nature of the primary cultures themselves. This year, we developed the tools and techniques required for primary culture of prostate cancer stem cells. Next year, we plan to establish primary cultures containing cells with stem cell characteristics, then return to our goal of identifying optimal techniques for tumor formation of these primary cultures *in vivo*. At the conclusion of our studies, we expect to provide a model system of *in vitro* and *in vivo* propagation of the cells most relevant to prostate cancer therapy, i.e., prostate cancer stem cells.

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APPENDICES

None.